## REMARKS

### *Claim Amendments*

Claim 1 has been amended to include the limitations of claims 2 and 3 and as described below.

Claims 2 and 3 have been canceled.

Claim 8 has been amended to change the recitation "a physiologically active ingredient" to "a pharmaceutically active ingredient". This amendment is supported by the description on page 31, lines 1-5, of the specification and by claims 2 and 3.

New claim 14 has been added to the application. New claim 14 is supported by the description on page 18, lines 3-5 and 9-12, of the present specification.

### *ACTION*

### *Claim Rejections - 35 USC § 112*

The Office is rejecting the claims as being indefinite under the second paragraph of 35 U.S.C. § 112 relating to the terminology "wild-type human serum albumin." The position of the Office is that this terminology would not be understood by a person of ordinary skill in the art because the specification does not define the terminology.

Applicants respectfully submit that the terminology "wild-type human serum albumin" is a term of art having an art-recognized

meaning. As evidence of this fact, applicants are submitting herewith abstracts from the following three prior art publications:

(1) Petersen, Charles E. et al., "Mutations in a Specific Human Serum Albumin Thyroxine Binding Site Define the Structural Basis of Familial Dysalbuminemic Hyperthyroxinemia", *The Journal of Biological Chemistry*, Vol. 271, No. 32, pp. 19110-19117 (1996);

(2) Liu, Ronya et al., "The Role of Electrostatic Interactions in Human Serum Albumin Binding and Stabilization by Halothane", *The Journal of Biological Chemistry*, Vol. 277, No. 39(?), pp. 36373-36379 (2002); and

(3) Yang, Jinsheng et al., "Structural insights into human serum albumin-mediated prostaglandin catalysts", *Protein Chemistry* (2002), 11; 538-545.

Moreover, the meaning of the terminology "wild type human serum albumin" recited in the claims is generally the same as that of "naturally occurring human serum albumin" and, as described in the present application, means albumin whose amino acid sequence is the same as that of "naturally occurring human serum albumin" (see, for example, page 18, lines 5-12, of the specification).

Applicants have also amended claims 1, 7 and 8 to recite that

the wild type human serum albumin is "genetically recombined wild type human serum albumin". This amendment is supported by the description on page 18, lines 10-12 of the specification, that "[i]n the present invention, it is preferred to use a genetically recombined albumin since there is no risk of infection.".

Removal of the 35 U.S.C. § 112, second paragraph, rejection is in order and is requested.

*Claim Rejections - 35 USC § 102/35 USC § 103(a)*

Claims 1-2, 5-6 and 8 are rejected under 35 U.S.C. 102(b) as being anticipated by Kamps et al. (*Biochimica et Biophysica Acta* 1278 (1996); hereinafter "Kamps"). Claims 1-13 are rejected under 35 U.S.C. 103(a) as being unpatentable over Tardi et al. (*J. Immunological Methods*, 1997; hereinatfer "Tardi"), alone, or in combination with Jacobsen (U.S. Patent Application Publication No. 2002/0132328) or Mayo (U.S. Patent Application Publication No. 2002/0146406). Claims 1-13 are rejected under 35 U.S.C. 103(a) as being unpatentable over Zalipski (U.S. Patent No. 6,180,134) in view of Kamps, optionally in combination with Jacobsen or Mayo, or over Kamps, optionally with Jacobsen or Mayo, in view of Zalipski.

The 35 U.S.C. § 102 rejection over Kamps is overcome by the amendment to claim 1 to recite that a pharmaceutically active ingredient is contained in the liposome.

Reconsideration of the 35 U.S.C. § 103(a) rejections is respectfully requested.

Tardi shows in Fig. 1 plasma elimination of PEG-liposomes with and without surface associated ovalbumin. It is clear from Fig. 1 that PEG-liposomes with surface associated ovalbumin are eliminated from the circulation at a rate faster than that observed for control liposomes (refer to "Results" beginning on page 141, left column). That is, the retention of liposomes with surface associated ovalbumin in the blood is distinctively lower than that of PEG-liposomes without surface associated ovalalbumin.

However, in the present invention, as shown in Fig. 1 of the present application, retention of liposomes in blood is significantly improved by bonding PEG and wild-type human serum albumin to liposomes, as compared when only PEG is bonded to liposome. The showing of Fig. 1, when considered with the teachings of Kamps, shows that, unexpectedly and contrary to the assertion in the Action, ovalbumin and wild-type human serum albumin are not equivalents and produce significantly different results when bonded to PEG. This showing rebuts the Office's cases of obviousness and overcomes the 35 U.S.C. § 103(a) rejections.

Removal of the 35 U.S.C. § 102 and 35 U.S.C. § 103(a) rejections is also in order.

A notice of allowability is respectfully requested.

The foregoing is believed to be a complete and proper response to the Office Action dated November 25, 2008

In the event that this paper is not considered to be timely filed, applicants hereby petition for an appropriate extension of time. The fee for any such extension and any additional required fees may be charged to Deposit Account No. 111833.

In the event any additional fees are required, please also charge our Deposit Account No. 111833.

Respectfully submitted,
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# Structural insights into human serum albumin-mediated prostaglandin catalysis

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**Abstract**

Previous studies have shown that many arachidonic acid metabolites bind to human serum albumin (HSA) and that the metabolism of these molecules is altered as a result of binding. The present study attempted to gain insights into the mechanisms by which prostaglandins bound to subdomain 2A of HSA are metabolized by catalytic processes. The breakdown of the prostaglandin 15-keto-$PGE_2$ to 15-keto-$PGA_2$ and 15-keto-$PGB_2$ in the presence of wild-type HSA and a number of subdomain 2A mutants was examined using a previously validated spectroscopic method which monitors absorbance at 505 nm. The species examined using this method were wild-type HSA, K195M, K199M, F211V, W214L, R218M, R218P, R218H, R222M, H242V, R257M, and bovine serum albumin. Previous studies of HSA-mediated catalysis indicated that the breakdown of HSA-bound prostaglandins results from an alkaline microenvironment in the binding site. Our results show that the catalytic breakdown of HSA-bound 15-keto-$PGE_2$ to 15-keto-$PGB_2$ results from two specific processes which are modulated by specific amino acid residues. Specifically, some amino acid residues modulate the rate of step 1, the conversion of 15-keto-$PGE_2$ to 15-keto-$PGA_2$, while other residues modulate the rate of step 2, the conversion of 15-keto-$PGA_2$ to 15-keto-$PGB_2$. Some residues modulate the rate of steps 1 and 2. In total, while our results support the involvement of certain basic amino acid residues in the catabolism of HSA-bound 15-keto-$PGE_2$, our data suggest that metabolism of HSA-bound prostaglandins may be a more complex and specific process than previously thought.

Keywords: Human serum albumin; prostaglandins; catalysis; binding site; site-directed mutagenesis

The structures of the first two prostaglandins, prostaglandins $E_1$ and $F_1$ ($PGE_1$ and $PGF_1$) were elucidated in 1962. As more prostaglandins were discovered it soon became clear that they all shared a similar chemical structure, namely they were 20-carbon unsaturated carboxylic acids with a cyclopentane ring, all of which were derived from the precursor arachidonic acid. It was soon found that arachidonic acid was a precursor for other chemically related biologically active molecules such as prostacyclin ($PGI_2$), thromboxanes, and leukotrienes. For a more complete background and synthesis pathways showing the interrelationships among the above compounds, the reader is referred to the pharmacology text by Campbell and Halushka (1996).

The general instability of prostaglandins and related compounds in aqueous media has complicated attempts to unravel the many biological roles played by these highly active signaling molecules. It became apparent early on in prostaglandin research that proteins in the blood might play an important role in modulating the biological activities of these compounds by binding to and stabilizing or destabilizing certain prostaglandins. A series of binding studies using radiolabeled $PGE_1$, $PGE_2$, $PGA_2$, and $PGF_2$ found that the only plasma protein that significantly binds to the above prostaglandins is human serum albumin (HSA) (Raz 1972). Although the affinity of HSA for a variety of bio-

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# Mutations in a Specific Human Serum Albumin Thyroxine Binding Site Define the Structural Basis of Familial Dysalbuminemic Hyperthyroxinemia*

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The familial dysalbuminemic hyperthyroxinemia (FDH) phenotype results from a natural human serum albumin (HSA) mutant with histidine instead of arginine at amino acid position 218. This mutation results in an enhanced affinity for thyroxine. Site-directed mutagenesis and a yeast protein expression system were used to synthesize wild type HSA and FDH HSA as well as several other HSA mutants. Studies on the binding of thyroxine to these HSA species using equilibrium dialysis and quenching of tryptophan 214 fluorescence suggest that the FDH mutation affects a single thyroxine binding site located in the 2A subdomain of HSA. Site-directed mutagenesis of HSA and thyroxine analogs were used to obtain information about the mechanism of thyroxine binding to both wild type and FDH HSA. These studies suggest that the guanidino group of arginine at amino acid position 218 in wild type HSA is involved in an unfavorable binding interaction with the amino group of thyroxine, whereas histidine at amino acid position 218 in FDH HSA is involved in a favorable binding interaction with thyroxine. Neither arginine at amino acid position 222 nor tryptophan at amino acid position 214 appears to favorably influence the binding of thyroxine to wild type HSA.

Familial dysalbuminemic hyperthyroxinemia (FDH),[1] an autosomal dominant condition in which the total thyroxine level in serum is elevated while the free thyroxine level is normal, results from the presence of an abnormal human serum albumin (HSA) with an enhanced affinity for thyroxine (1). Although this condition had been widely reported in the medical literature (1–8), the molecular basis of FDH was not known until the identification of a single point mutation in the HSA gene of several FDH individuals resulting in the substitution of histidine for arginine at amino acid position 218 (9). This result was confirmed by another study in which the same mutation was identified in FDH individuals from eight unrelated families (10). Recently, it was shown that recombinantly produced FDH HSA has an enhanced affinity for thyroxine similar to that seen for natural FDH HSA (11), a result that confirmed that all of the information necessary to generate the FDH phenotype is contained in the FDH mutation.

The binding of thyroxine to HSA has been extensively studied (12–23), yet the molecular basis of this interaction remains obscure. Early studies used equilibrium dialysis to measure the binding of radiolabeled thyroxine and radiolabeled thyroxine analogs to HSA. Interpretation of these results was complicated by the observation of several binding components, which were difficult to resolve. For example, some of these studies assigned four equal binding sites for thyroxine with dissociation constants ($K_d$) of 6.6 $\mu$M (12–14), whereas other studies resolved the binding data into two sites with $K_d$ values of 3.6 $\mu$M and six sites with $K_d$ values of 25 $\mu$M (15–16). Data from other equilibrium dialysis studies were interpreted as fitting best to a multi-site model with one high affinity site ($K_d$ value of 0.83 $\mu$M) and six lower affinity sites ($K_d$ values of 15 $\mu$M) (17).

The aforementioned results indicated that HSA has multiple thyroxine binding sites, whereas the existence of a specific thyroxine binding site in the 2A subdomain of HSA was suggested by other observations. Specifically, the 2A subdomain has been shown to be one of the two principal binding sites on HSA for small hydrophobic ligands (24–27). Binding studies with proteolytic HSA fragments have shown that the high affinity bilirubin binding site of HSA is located in the 2A subdomain (28). Other studies have shown that thyroxine competes with bilirubin binding at this high affinity bilirubin binding site, suggesting that the sites for these two ligands overlap (29).

HSA contains a single tryptophan residue at amino acid position 214, which is located in the 2A subdomain, and the fluorescence of this tryptophan is quenched by the binding of thyroxine (23, 30). This quenching has been exploited to measure the binding of thyroxine (23, 30), bilirubin (31), and a number of other 2A ligands (32–34). Studies measuring thyroxine binding to HSA by the fluorescence quenching method indicated a single high affinity site with a $K_d$ value of 0.63 $\mu$M (23), in close agreement with the high affinity site ($K_d$ of 0.83 $\mu$M) determined from equilibrium dialysis experiments (17).

To improve our understanding of the mechanism of thyroxine binding to the 2A subdomain of HSA, we used site-directed mutagenesis and a protein expression system to synthesize wild type HSA, FDH HSA, and several HSA mutants. The fluorescence quenching technique was used to measure the binding affinity of thyroxine and the thyroxine analogs, tetraiodothyroacetic acid (TA), 3,5,3′-triiodothyronine (T3), 3,5,3′-triiodothyropropionic acid (TP), and 3,3′,5′-triiodothyronine (RT3) to wild type HSA and to the HSA mutants (with the exception of a mutant in which leucine was substituted for tryptophan). The binding of thyroxine to wild type HSA and to the mutants was also measured by equilibrium dialysis. The following HSA mutants were synthesized: R218H (FDH) and R218M HSA substituting histidine or methionine for arginine at amino acid position 218, respectively; W214L HSA substi-

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[1] The abbreviations used are: FDH, familial dysalbuminemic hyperthyroxinemia; HSA, human serum albumin; PBS, phosphate-buffered saline; TA, tetraiodothyroacetic acid; T3, 3,5,3′-triiodothyronine; TP, 3,5,3′-triiodothyropropionic acid; RT3, 3,3′,5′-triiodothyronine.

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# The Role of Electrostatic Interactions in Human Serum Albumin Binding and Stabilization by Halothane*

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**Electrostatic interactions have been proposed as a potentially important force for anesthetics and protein binding but have not yet been tested directly. In the present study, we used wild-type human serum albumin (HSA) and specific site-directed mutants as a native protein model to investigate the role of electrostatic interactions in halothane binding. Structural geometry analysis of the HSA-halothane complex predicted an absence of significant electrostatic interactions, and direct binding (tryptophan fluorescence and zonal elution chromatography) and stability experiments (hydrogen exchange) confirmed that loss of charge in the binding sites, by charged to uncharged mutations and by changing ionic strength of the buffer, generally increased both regional (tryptophan region) and global halothane/HSA affinity. The results indicate that electrostatic interactions (full charges) either do not contribute or diminish halothane binding to HSA, leaving only the more general hydrophobic and van der Waals forces as the major contributors to the binding interaction.**

Inhalational anesthetics can alter the activity of a wide variety of proteins, but the molecular nature of the interactions underlying the functional effect is still poorly understood. Guided by the Meyer-Overton correlation between anesthetic potency and solubility in a lipid-like environment, studies in the past three decades have concluded that anesthetics must bind to hydrophobic regions within target protein (especially membrane protein, i.e. ion channels) through weak van der Waals interactions and the hydrophobic effect (1–5). Electrostatic interactions were proposed recently as potentially important binding forces between anesthetics and target proteins (1, 6, 7).

Halogen atoms, especially fluorine, are more electronegative than carbon atoms, and therefore the C-halogen bond is polarized in inhalational anesthetics. *Ab initio* calculations indicated that halothane has a small permanent dipole moment (8), which may contribute binding to relevant targets. Similar compounds with less dipole than halothane are poor anesthetics, although this might be partially due to much lower solubility in water (1). Because polarity appears to be an important feature of anesthetics, it is reasonable to speculate that anesthetic binding sites contain polar moieties. Charged residues such as arginine and lysine and polar but uncharged aromatic groups with a partial negative charge in the center of the ring (9, 10) may contribute to the polarity of anesthetic binding sites. The weakly polar anesthetics might therefore interact with the charged residues directly and/or form dipole-quadrupole (a form of weak cation-π) interactions with the aromatic side chains. The latter may be strengthened by the positively charged residues coordinating the more electronegative end of the anesthetic molecule.

Because functionally important *anesthetic targets* remain unidentified, we have made use of surrogate proteins with appropriate binding character (1, 11–14). Designed peptides, for example, have been used to investigate the halothane binding site and its characteristics. In a synthetic four-helix-bundle protein, substitution of tyrosine for tryptophan decreased anesthetic binding affinity by about 5-fold, suggesting that the less dense electron cloud of tyrosine coordinates the relatively positive end of the anesthetic molecule less well (14). In the present study, we have used human serum albumin (HSA)[1] as a native protein model to investigate electrostatic interactions directly. HSA is useful because it satisfies major pharmacodynamic criteria for simulating the anesthetic targets (11) and has a binding affinity for halothane within 10-fold of its clinical $EC_{50}$, and a high resolution structure of HSA alone and in complex with halothane is now available (15). We focused on a large interdomain cavity containing the only tryptophan in HSA in this study. Previous work has confirmed that anesthetics bind to this region (12, 16), which also contains many charged residues (15, 17). If the relatively positive end of halothane coordinates with the π system of the tryptophan indole ring in a weak cation-π interaction, then it is possible that nearby positively charged residues may coordinate the relatively negative trifluoromethyl end of halothane (1). We predict that loss of positively charged side chains near Trp will eliminate an electrostatic contribution to halothane binding and therefore weaken halothane-HSA binding constants. To test this, we performed geometrical analysis of HSA (1E7B) and the HSA-halothane complex structures (1E7B and 1E7C), expressed nine site-directed HSA mutants, and tested these for altered halothane binding using fluorescence spectroscopy, zonal elution chromatography, and amide hydrogen exchange combined with ionic strength experiments.

## EXPERIMENTAL PROCEDURES

### *Materials*

Halothane (1-bromo-1-chloro-2,2,2-trifluoroethane) was obtained from Halocarbon Laboratories (Hackensack, NJ). The thymol preserv-

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[1] The abbreviations used are: HSA, human serum albumin; wtHSA, wild-type HSA.